Development of ELISA Assays to Detect Cry9C-specific IgG and IgE Antibodies in Human Serum

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The assay development study presented herein is not subject to the 40 CFR 160 Good Laboratory Practice (GLP) Standards. Standard operating procedures (SOPs) for this assay were part of the development process. GLPs and SOPs for instrument maintenance and operation, record keeping, documentation, personnel training were followed.

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SUMMARY

ELISA systems were developed to measure the levels of Cry9C-specific IgG antibody present in goat and rabbit polyclonal antibody preparations provided by Aventis CropScience. Background Cry9C cross-reactivity was observed in both rabbit and goat normal sera obtained from commercial vendors. In both species, this cross-reactivity was approximately 1000-fold less than that obtained from the corresponding polyclonal preparations. The human ELISA for Cry9C-reactive human IgG also had background reactivity to Cry9C. The level of cross-reactivity was variable and was comparable (means within 2 Standard Deviations) for both pre-1998 and post-1998 human serum. Low levels of cross-reactivity were also measured in nut-allergic sera.

The development of the Cry9C-specific IgE ELISA was preceded by the development of an ELISA for total serum IgE levels. The total IgE ELISA was used to demonstrate binding of the anti-human IgE conjugate to IgE in normal serum and assay sensitivity. The total IgE ELISA was a sensitive assay able to detect less than 1 ng/mL of human IgE with good reproducibility (both inter-assay, and intra-assay) and good linearity over at least a 10-fold dilution range. Total IgE titers were obtained for the panel of pre-1998 and post-1998 sera, and for nut-allergic sera. Using the system optimized for the total IgE ELISA, and the Cry9C coating concentrations used to detect Cry9C-specific IgG, an initial panel of pre- and post-1998 samples was evaluated for both total IgE and Cry9C-specific IgE. Total IgE titers were detectable in all samples tested, in a range of concentrations and in agreement with published values for total IgE. There was no IgE Cry9C-reactivity in any of the samples tested.

In summary, ELISA assays were developed to detect Cry9C-specific IgG antibody, Cry9C-specific IgE antibody, and total IgE antibody, using a small number of pre-1998 and post-1998 human samples. Additional work is in progress to use these assay systems to measure Cry9C-specific IgG, Cry9c-specific IgE, and total IgE in 100 (pre-1998) plasma samples received from the American Red Cross.

1.0 OBJECTIVE

The objective of this project was the development of enzyme-linked immunoassays (ELISA) to detect Cry9C-specific IgG, Cry9C-specific IgE, and total IgE antibodies in human serum. This work was also recommended by the December 1, 2000 FIFRA Scientific Advisory Panel report

2.0 EXPERIMENTAL APPROACH

The experimental approach was to first develop systems to measure the levels of Cry9C-specific IgG antibody present in goat and rabbit polyclonal antibody preparations provided by Aventis CropScience. These systems with known positives (polyclonal serums) would allow for the determination of required concentration of the coating antigen (Cry9C) and initial assay development with respect to assay buffers and substrate systems. The human ELISA development (where no positive control is available to date) would be based on the goat and rabbit data and evaluation of a serum sample panel containing both 1995 (pre-1998) and 2001 (post-1998) serum samples.

The development of the Cry9C-specific IgE ELISA was preceded by the development of an ELISA for total serum IgE levels. The total IgE ELISA was used to demonstrate binding of the anti-human IgE conjugate to IgE in normal serum and assay sensitivity. Using the system optimized for the total IgE ELISA, and the Cry9C coating concentrations used to detect Cry9C-specific IgG, an initial panel of pre- and post-1998 samples was evaluated for both total IgE and Cry9C-specific IgE.

Serum from 5 peanut-allergic individuals and 1 Brazil nut-allergic individual were kindly provided by Dr. Susan Hefle, University of Nebraska, and were evaluated for Cry9C-specific IgG, Cry9C-specific IgE, and total IgE

3.0 METHODOLOGY

In these assays, a microtiter (96-well) flat-bottom plate was coated with the antigen (or antibody) of interest and allowed to incubate overnight at 4°C. The coating material was aspirated and the plate was blocked for 1 hour with a buffer solution containing bovine serum albumin (BSA). Following the blocking step, the block buffer was aspirated and the primary antibody (or sample) that was being tested for reactivity (binding) with the coated material, was added. The plate was

incubated to allow binding to occur. Upon incubation, the antibody/sample was aspirated, and the plate washed to remove any unbound antibody/sample. The conjugated antibody was then added to the plate and allowed to incubate for 1 hour. Upon incubation, the conjugate was aspirated, the plate was washed (to remove any unbound conjugated antibody), and a substrate was added. The substrate used (TMB, or Blue Phos) was dependent on whether peroxidase or alkaline phosphate was conjugated to the secondary antibody. The colorimetric reaction was allowed to develop for 30 minutes and was stopped after that time with a stopping agent (specific for the detection system being used). The actual absorbance reading (the appropriate absorbance depends on the detection system being used) of the individual wells was obtained using a Spectramax 340 (Molecular Devices) microplate reader and SoftmaxPro v.2.2.1 software (Molecular Devices).

3.1 ELISA titer determinations

Total IgE:

All data manipulations were performed using SoftmaxPro. Average blank optical density (OD) readings (block buffer only) were subtracted from all standard and sample wells to obtain a corrected OD value. A standard curve was generated by plotting the mean corrected OD value for duplicate standards vs. concentration using a four parameter logistic (4-PL) curve fit. Total IgE titers were obtained by interpolation from the standard curve and multiplication by the dilution factor.

Cry9C-specific ELISA:

Using SoftmaxPro, average blank OD readings (block buffer only) were subtracted from all sample wells OD to obtain a corrected OD value. For each sample, the dilution factor for each dilution was plotted vs. its corresponding corrected OD value using a log-log curve fit. An acceptable sample curve was defined by a minimum of 4 data points with an R^2 value ≥ 0.98. Using Microsoft Excel, the titer was defined in arbitrary ELISA Units (EU) as the dilution factor that corresponded to an OD of three (3) times background (mean blank OD).

Table 1 below summarizes the assays developed. Assay specifics may be found in the 'Materials and Reagents' and 'Procedure' sections for the individual ELISA.

Table 1- ELISA Development Summary

Objective	Coat	Block	Primary Ab/sample	Wash	Secondary Ab	Wash	Substrate	Stop
Determine levels of circulating Cry9C-specific IgG in normal goat serum and in goats immunized with Cry9c	CRY 9C	√	Normal goat serum or goat α-Cry9C serum	√	Rabbit α-goat IgG Peroxidase-labeled or Alkaline Phosphatase-(AP) labeled	√	TMB or Blue Phos	(a)1N H_2SO_4 OR (b)2.5% EDTA
Determine levels of circulating Cry9C-specific IgG in normal rabbit serum and in rabbits immunized with Cry9C	CRY 9C	√	Normal rabbit serum or rabbit α-Cry9C serum	√	Goat α-rabbit IgG AP-labeled	√	Blue Phos	2.5% EDTA
Determine levels of circulating Cry9C-specific IgG in human serum samples	CRY 9C	√	Human serum samples	√	Goat α-human IgG AP-labeled	√	Blue Phos	2.5% EDTA
Determine levels of circulating Cry9C-specific IgE in human serum samples	CRY 9C	√	Human serum samples	√	Goat α-human IgE AP-labeled	√	Blue Phos	2.5% EDTA
Determine levels of circulating Total IgE in human serum samples (against a standard curve of purified human IgE)	Monoclonal Ab to Human IgE	√	Human serum samples or purified human IgE	√	Goat α-human IgE AP-labeled	√	Blue Phos	2.5% EDTA

Ab = Antibody
TMB = tetramethylbenzidine
EDTA=ethylenediamine tetraacetic acid

3.2 Goat Cry9C-specific IgG ELISA

Materials and Reagents:

- High binding ELISA microplates (Greiner)
- Coating antigen (5 ug/mL Cry9C in Phosphate Buffered Saline (PBS))
- Goat anti-Cry9C polyclonal preparation (Aventis)
- Normal goat serum (Sigma)
- Rabbit anti-Goat IgG Peroxidase-labeled (Sigma)- 1:30,000 in block buffer)
- Rabbit anti-Goat IgG Alkaline Phosphatase-labeled (Sigma) –1:10,000 in block buffer
- Block Buffer (PBS / 0.05% Tween 20 / 0.1% BSA)
- Wash Buffer (PBS / 0.05% Tween 20)
- Tetramethylbenzidine (TMB) (KPL)

- 1N Sulfuric acid (Mallincrodt)- Stop solution for TMB
- Blue Phos (KPL)
- Blue Phos Stop Solution (2.5% EDTA) (KPL)

Procedure:

1. Coat the microwell plate with 0.1 mL/well of Cry9C (5ug/mL). Cover and place at 4°C overnight.
2. Add block buffer (200 uL/well)
3. Incubate for 1 hour at room temperature
4. Aspirate block buffer and blot plate
5. Add sample (normal goat serum sample or goat polyclonal antibody) (100 uL/well)
6. Cover plate and incubate for 1 hour at room temperature
7. Aspirate, wash plate 4 times with wash buffer, and blot dry
8. Add rabbit anti-goat conjugate: (100 uL/well)
9. Cover plate and incubate for 1 hour at room temperature
10. Aspirate, wash plate 4 times with wash buffer, and blot dry
11. Add substrate (TMB for peroxidase, or Blue Phos for alkaline phosphatase): 100 uL/well
12. Incubate for 30 minutes at room temperature
13. Add Stop solution (100 uL/well)
14. Read absorbance: A-450 for peroxidase/TMB/Sulfuric acid or A-650 for Alkaline phosphatase/Blue Phos/2.5% EDTA

3.3 Rabbit Cry9C-specific IgG ELISA

Materials and Reagents:

- High binding ELISA microplates (Greiner)
- Coating antigen (5 ug/mL Cry9C in Phosphate Buffered Saline (PBS))
- Rabbit anti-Cry9C polyclonal preparation (Aventis)
- Normal rabbit serum (Sigma)
- Goat anti-rabbit IgG Alkaline Phosphatase-labeled (Sigma) – 1:20,000 in block buffer
- Block Buffer (PBS / 0.05% Tween 20 / 0.1% BSA)
- Wash Buffer (PBS / 0.05% Tween 20)
- Blue Phos (KPL) - Substrate
- Blue Phos Stop Solution (2.5% EDTA) (KPL)

Procedure:

1. Coat the microwell plate with 0.1 mL/well of Cry9C (5ug/mL). Cover and place at 4°C overnight.
2. Add block buffer (200 uL/well)
3. Incubate for 1 hour at room temperature
4. Aspirate block buffer and blot plate
5. Add sample (normal rabbit serum sample or rabbit polyclonal antibody to Cry9C) (100 uL/well)
6. Cover plate and incubate for 1 hour at room temperature
7. Aspirate, wash plate 4 times with wash buffer, and blot dry
8. Add goat anti-rabbit conjugate: (100 uL/well)
9. Cover plate and incubate for 1 hour at room temperature
10. Aspirate, wash plate 4 times with wash buffer, and blot dry
11. Add substrate (100 uL/well)
12. Incubate for 30 minutes at room temperature
13. Add Stop solution (100 uL/well)
14. Read absorbance: A-650

3.4 Human Cy9C-specific IgG ELISA

Materials and Reagents:

- High binding ELISA microplates (Greiner)
- Coating antigen (5 ug/mL Cry9C in Phosphate Buffered Saline (PBS))
- Normal Human Serum minus IgG (Sigma) – control
- Human serum samples
- Goat anti-human IgG Alkaline Phosphatase-labeled (Sigma) – 1:5,000 in block buffer
- Block Buffer (PBS / 0.05% Tween 20 / 0.1% BSA)
- Wash Buffer (PBS / 0.05% Tween 20)
- Blue Phos (KPL) - Substrate
- Blue Phos Stop Solution (2.5% EDTA) (KPL)

Procedure:

1. Coat the microwell plate with 0.1 mL/well of Cry9C (5ug/mL). Cover and place at 4°C overnight.
2. Add block buffer (200 uL/well)
3. Incubate for 1 hour at room temperature
4. Aspirate block buffer and blot plate
5. Add sample (100 uL/well)
6. Cover plate and incubate for 1 hour at room temperature
7. Aspirate, wash plate 4 times with wash buffer, and blot dry
8. Add goat anti-human IgG conjugate: (100 uL/well)
9. Cover plate and incubate for 1 hour at room temperature
10. Aspirate, wash plate 4 times with wash buffer, and blot dry
11. Add substrate (100 uL/well)
12. Incubate for 30 minutes at room temperature
13. Add Stop solution (100 uL/well)
14. Read absorbance: A-650

3.5 Human Total IgE ELISA

Materials and Reagents:

- High binding ELISA microplates (Greiner)
- Capture antibody: Monoclonal anti-human IgE, clone GE-1 (Sigma) in Phosphate Buffered Saline (PBS) – 10 ug/mL
- Standard: Purified human IgE (Binding Site) 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 ng/mL in block buffer
- Human serum samples diluted 1:10 in block buffer
- Goat anti-human IgE Alkaline Phosphatase-labeled (Sigma) – 1:500 in block buffer
- Block Buffer (PBS / 0.05% Tween 20 / 0.1% BSA)
- Wash Buffer (PBS / 0.05% Tween 20)
- Blue Phos (KPL) - Substrate
- Blue Phos Stop Solution (2.5% EDTA) (KPL)

Procedure:

1. Coat the microwell plate with 0.1 mL/well of the capture antibody. Cover and place at 4°C overnight.
2. Add block buffer (200 uL/well)
3. Incubate for 1 hour at room temperature

4. Aspirate block buffer and blot plate
5. Add sample / standards(100 uL/well)
6. Cover plate and incubate for 1 hour at room temperature
7. Aspirate, wash plate 4 times with wash buffer, and blot dry
8. Add goat anti-human conjugate: (100 uL/well)
9. Cover plate and incubate for 1 hour at room temperature
10. Aspirate, wash plate 4 times with wash buffer, and blot dry
11. Add substrate (100 uL/well)
12. Incubate for 30 minutes at room temperature
13. Add Stop solution (100 uL/well)
14. Read absorbance: A-650

3.6 Human Cry9C-specific IgE ELISA

Materials and Reagents:

- High binding ELISA microplates (Greiner)
- Coating antigen (5 ug/mL Cry9C in Phosphate Buffered Saline (PBS))
- Goat anti-human IgE Alkaline Phosphatase-labeled (Sigma) – 1:500 in block buffer
- Human serum samples diluted in block buffer
- Block Buffer (PBS / 0.05% Tween 20 / 0.1% BSA)
- Wash Buffer (PBS / 0.05% Tween 20)
- Blue Phos (KPL) - Substrate
- Blue Phos Stop Solution (2.5% EDTA) (KPL)

Procedure:

1. Coat the microwell plate with 0.1 mL/well of Cry9C (5ug/mL). Cover and place at 4°C overnight.
2. Add block buffer (200 uL/well)
3. Incubate for 1 hour at room temperature
4. Aspirate block buffer and blot plate
5. Add sample (100 uL/well)
6. Cover plate and incubate for 1 hour at room temperature
7. Aspirate, wash plate 4 times with wash buffer, and blot dry
8. Add goat anti-human conjugate: (100 uL/well)
9. Cover plate and incubate for 1 hour at room temperature
10. Aspirate, wash plate 4 times with wash buffer, and blot dry
11. Add substrate (100 uL/well)
12. Incubate for 30 minutes at room temperature
13. Add Stop solution (100 uL/well)
14. Read absorbance: A-650

4.0 RESULTS

4.1 Goat anti-Cry9C IgG

Evaluation of Coating Concentrations of Cry9C

Different coating concentrations of Cry9C in phosphate buffered saline (PBS) (0.5, 0.75, and 1 ug/well) were evaluated. Dilutions of the polyclonal goat anti-Cry9C were used (1:100 serially diluted 1:2 in block buffer). The conjugate was rabbit anti-goat IgG peroxidase labeled at 1:50,000. Equivalent signal was

detected at the three concentrations tested and further experiments were performed with the 0.5 ug/well (5 ug/mL) coating concentration.

Evaluation of Peroxidase-labeled Rabbit Anti-Goat Conjugate

Using a 0.5 ug/well coating concentration of Cry9C and dilutions of the goat anti-Cry9C polyclonal (1:1000 serially diluted 1:2) plated in duplicate, different dilutions of the peroxidase-labeled conjugate were evaluated. The results may be seen in Table 2 below. Titers were reported in arbitrary ELISA Units (EU), as previously defined in the Methodology section of this report.

Table 2: Evaluation of dilutions of anti-goat peroxidase conjugate

Rabbit anti-goat IgG peroxidase-labeled	Titer (EU)	Mean Plate Blank
1:10000	1,115,918	0.081
1:20000	901,195	0.067
1:30000	644,479	0.065
1:50000	46,144	0.057

Evaluation of Alkaline Phosphatase-labeled Rabbit Anti-Goat Conjugate

Using a 0.5 ug/well coating concentration of Cry9C and dilutions of the goat anti-Cry9C polyclonal (1:2000 serially diluted 1:2) plated in duplicate, different dilutions of the alkaline phosphatase-labeled rabbit anti-goat conjugate were evaluated. The results may be seen in Table 3 below. Titers were reported in arbitrary ELISA Units (EU), as previously defined in the Methodology section of this report. The sensitivity was approximately two-fold lower than for the peroxidase conjugate but still resulted in excellent signal detection, with the added advantage of very low background (blank) absorbance values.

Table 3: Evaluation of dilutions of anti-goat alkaline phosphatase (AP) conjugate

Rabbit anti-goat IgG AP-labeled	Titer (EU)	Mean Plate Blank
1:10000	465,045	0.038
1:40000	94,179	0.042

Evaluation of Normal Goat Serum Reactivity to Cry9C

Dilutions of normal goat serum (serially diluted 1:2) and of the goat anti-Cry9C polyclonal were evaluated in the same assay. The ELISA was performed as described in the Methodology section using 0.5 ug/well of Cry9C, serial dilutions of the samples to be tested, and a 1:10,000 dilution of the AP-conjugated rabbit anti-goat IgG antibody. Normal goat serum does exhibit some reactivity (537 EU) to the Cry9C. This background cross-reactivity is approximately 1000-fold lower than for the goat anti-Cry9C polyclonal antibody (465,045 EU).

4.2 Rabbit anti-Cry9C IgG

Evaluation of Alkaline Phosphatase-labeled Goat Anti-Rabbit Conjugate

Using a 0.5 ug/well coating concentration of Cry9C and dilutions of the rabbit anti-Cry9C polyclonal (1:1000 serially diluted 1:2) plated in duplicate, different dilutions of the alkaline phosphatase-labeled goat anti-rabbit conjugate were evaluated. The results may be seen in Table 4 below. Titers were reported in arbitrary ELISA Units (EU), as previously defined in the Methodology section of this report. Increased sensitivity could be obtained with increasing concentration of the conjugate with minimal to no effect on the background blank value. The 1:20,000 dilution of conjugate was selected as it provided good signal with starting absorbance values in the convenient absorbance range of about 2.0 (at the 1:1000 dilution of the polyclonal).

Table 4: Evaluation of dilutions of anti-rabbit AP conjugate

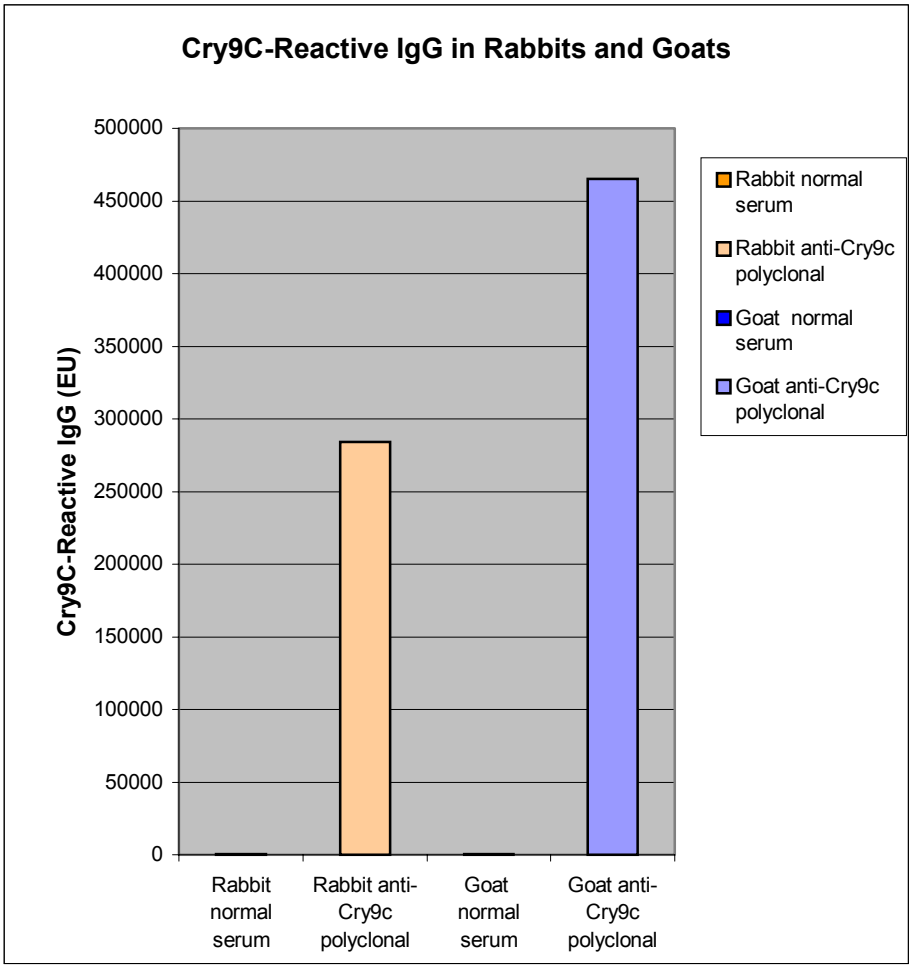
Goat anti-rabbit IgG AP-labeled	Titer (EU)	Mean Plate Blank
1:5000	768,504	0.040
1:10000	438,766	0.038
1:20000	205,511	0.038
1:30000	117,252	0.037

Evaluation of Normal Rabbit Serum Reactivity to Cry9C

When normal rabbit serum and the rabbit polyclonal antibody were evaluated in the same assay, the rabbit serum also exhibited some reactivity to Cry9C. However, as with the goat system, the cross-reactivity was approximately 1000-fold less than that exhibited by the polyclonal rabbit antibody (289 EU vs. 283,923

EU). The goat and rabbit data are summarized in Figure 1. The cross-reactivity baseline is barely visible in this graph because of the 1000-fold difference between the normal serum and the polyclonal preparations.

Figure 1 : Cry9C-Reactive IgG in Rabbits and Goats



4.3 Human anti-Cry9C IgG

Evaluation of Normal Human Serum Reactivity to Cry9C

The microplates were coated with Cry9C at 0.5 ug/well, and initial experiments had demonstrated similar background Cry9C-reactivity in human serum samples with a conjugate (goat anti-human IgG, AP-labeled) at a 1:5000. As with the goat and rabbit systems, this reactivity was observed in human serum samples whether pre- or post-1998. It was also observed in purified human IgG preparations (Sigma). No reactivity was seen when normal human serum with IgG absorbed (Sigma) was used. These samples were run either singly (304, 2425, and purified IgG) or in duplicate (human serum minus IgG) with serial dilution starting neat or at a 1:2 (purified IgG only). Each sample was run through an eleven (11) two-fold dilution series. The resulting titers are summarized in Table 5. All titers are expressed as ELISA Units (EU) and were determined as described in the Methodology section.

Table 5: Cry9C-reactive IgG in Human Serum Samples

Sample	Titer (EU)
Pre-1998 (303)	267
Post-1998 (2425)	98
Human IgG	58
Human serum minus IgG	(*)

(*) Not Detectable

Inter-assay variability

To evaluate inter-assay variability, five (5) human serum samples were run in duplicate on three different days. Samples were chosen based upon the volume available. The assay was performed as previously described in the Methodology section with samples serially diluted (starting at a 1:10) and tested in duplicates, and a conjugate dilution of 1:5000. The Cry9C-reactive titers for these four assays are shown in Table 6 below. The CV% reflects the variation that exists from creating a regression line on a series of points and interpolating the dilution corresponding to a pre-determined value. These CV% are not unusual given the low numbers of assays evaluated (3 assay runs) due to the limited availability of pre-1998 sera. Ongoing analysis of 100 American Red Cross pre-1998 samples across multiple assays should greatly reduce the inter-assay CV%.

Table 6 : Cry9C-reactive Human IgG

Sample #	Run 1	Run 2	Run 3	Mean EU	SD	CV%
2498	11.98	21.61	14.68	16.09	4.97	30.87
2499	38.28	50.08	51.60	46.65	7.29	15.63
2500	15.52	11.43	14.14	13.70	2.08	15.19
2501	23.31	18.32	19.83	20.49	2.56	12.48
2502	868.50	1282.73	1225.87	1125.70	224.55	19.95

Intra-assay variability

To evaluate intra-assay variability, one human serum sample was serially diluted and run in duplicate in 6 different replicates. The assay was performed as previously described in the Methodology section, with samples serially diluted and tested at a starting dilution of 1:10 in block buffer, and a conjugate dilution of 1:5000. The Cry9C-reactive IgG titers are shown in Table 7 below. The assay exhibited excellent intra-assay reproducibility with CV% = 6%.

Table 7: Intra-Assay Variability for Cry9C-reactive IgG

Sample #	Titer (EU)	Mean	SD	CV%
1-a	14.8	13.5	0.8	6.0
1-b	13.6			
1-c	14.1			
1-d	12.8			
1-e	12.9			
1-f	13.0			

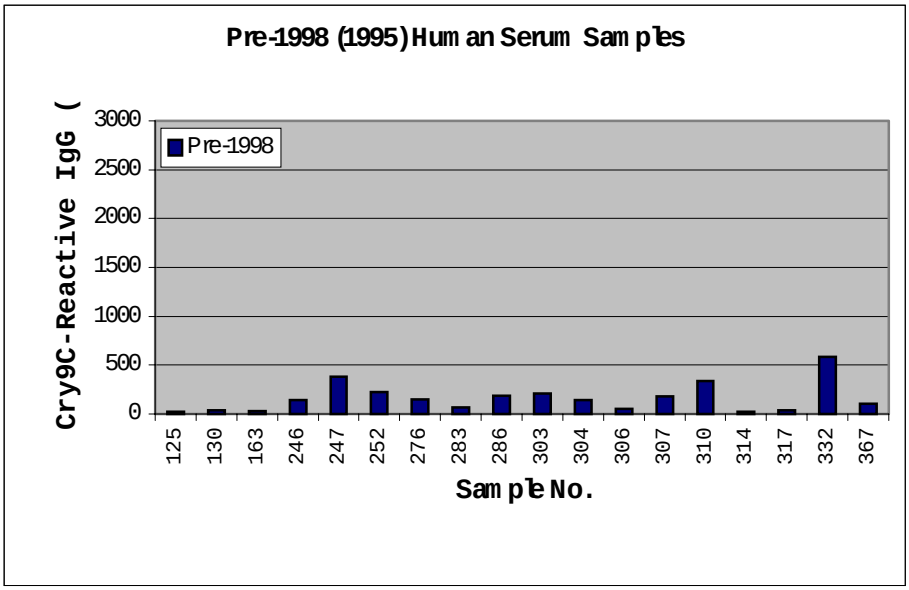
Initial screen of a panel of pre- and post-1998 human serum samples

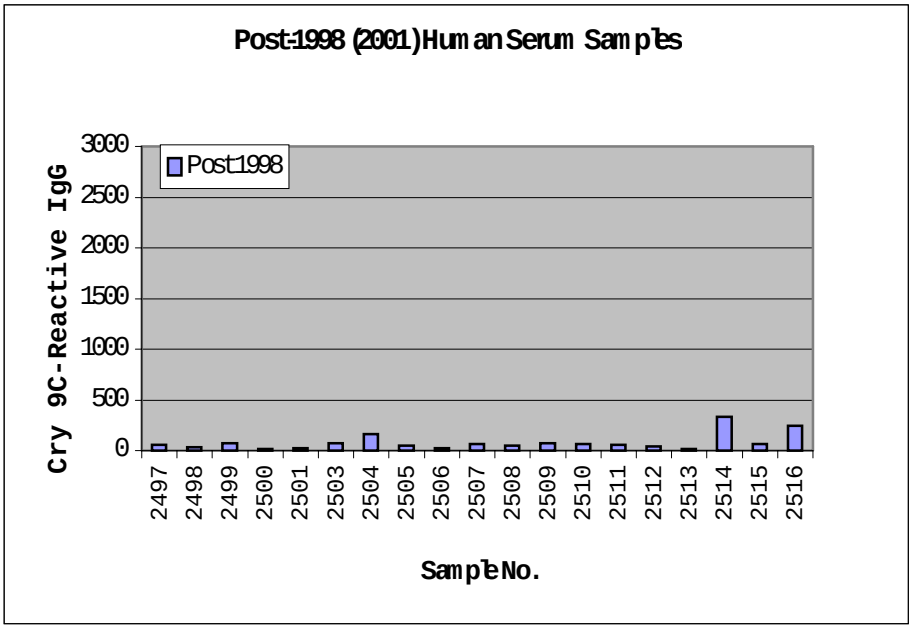
An initial screen of pre-1998 and post-1998 human serum samples was performed. These samples were available in the laboratory and had been used for validation of other ELISA assays, or tested as clinical samples. All samples were serially diluted and run singly. As can be seen in Table 8 and Figure 2, both groups exhibited similar variable reactivities. For neither group were any of the titers above 600 EU. The group means were 163 EU and 80 EU for Pre-1998 and Post-1998 serum samples respectively.

Table 8 : Cry9C-Reactive IgG – Initial Sample Screen

Pre-1998			Post-1998	
Sample No.	ELISA units		Sample No.	ELISA units
125	22		2497	60
130	40		2498	30
163	28		2499	74
246	146		2500	17
247	381		2501	24
252	227		2503	71
276	153		2504	164
283	68		2505	52
286	185		2506	28
303	211		2507	63
304	146		2508	50
306	49		2509	70
307	179		2510	62
310	339		2511	53
314	22		2512	44
317	40		2513	15
332	587		2514	337
367	104		2515	68
-	-		2516	240
Mean:	163		Mean:	80
SD:	149		SD:	82
SE:	35		SE:	19

Figure 2: Cry9C-Reactive IgG – An Initial Screen of Human Serum Samples





Evaluation of Peanut and Brazil Nut Allergic Individuals

Serum samples from five (5) peanut allergic, and one (1) brazil nut allergic individuals were provided by Dr. S. Hefle from the University of Nebraska. Each sample was serially diluted and tested in duplicate. The assay and all data were performed as described in the Methodology section. The titers obtained (Table 9) ranged from 20-86 and were all within the range of the previously screened pre-1998 samples.

Table 9: Cry9C-Reactive IgG in Individuals with Nut Allergies

Sample No.	Cry9C-Reactive IgG (EU)
1P	37
2	32
3	86
4	32
5	24
6	20

4.4 Human Total IgE

Evaluation of different coating concentrations

The monoclonal anti-human IgE antibody was coated on microplates at a concentration of 0.5, 1, and 2 ug/well in PBS and the plates incubated overnight at 4°C. The plates were blocked for 1 hour with block buffer. Purified IgE standard was serially diluted and added in duplicate at concentrations ranging from 4.9-5000 ng/mL. The alkaline phosphatase labeled goat anti-human IgE was diluted 1:1000 in block buffer and 100uL was added to each well. All standard and conjugate dilutions were performed in block buffer.
A coating concentration of 1 ug/well resulted in absorbance values comparable to those of the 2 ug/well, but slightly better those for the 0.5 ug/well plate. All three standard curves had R^2 values >0.99.

Evaluation of different conjugated antibody concentrations

The monoclonal anti-human IgE antibody was coated on microplates at a concentration of 1ug/well in PBS. The purified IgE standard was serially diluted and added in duplicate at concentrations ranging from 0.488-5000 ng/mL. The alkaline phosphatase labeled goat anti-human IgE was diluted 1:500, 1:1000, and 1:2000 in block buffer and 100uL was added to each well. All standard and conjugate dilutions were performed in block buffer. Maximal sensitivity was obtained with the 1:500 conjugate dilution.

Linearity of Sample Dilutions in Block Buffer

Using a coating concentration of 1 ug/mL, a conjugate dilution of 1:500, and a standard curve range of 0.488 - 500 ng/mL, three (3) human serum samples were serially diluted 1:10 – 1:10,240 and plated in duplicate.

For each sample the mean IgE value obtained from the sample duplicates was plotted vs. the dilution (reciprocal of the dilution factor). A linear regression was performed and the R^2 value obtained. The %CV between individual dilution of the same sample was also calculated. This data are presented in Table 10. The three samples had excellent linearity across the 1:10 to 1:160 dilution range. All three samples had correlation coefficients (R^2 values) greater than 0.999 and CV% ranging from 2.3 - 18.9%.

Table 10: Linearity of Sample Dilution (a)

Sample	Mean IgE (ng/mL)	Dilution	R2 Value	Mean IgE (ng/mL)	Dilution factor	Adjusted IgE (ng/mL)	Mean	SD	CV%
2489	20.5	0.1	0.9995	20.5	10	204.6	218.8	28.1	12.8
	10.3	0.05		10.3	20	205.8			
	5.0	0.025		5.0	40	199.1			
	2.7	0.0125		2.7	80	216.7			
	1.7	0.00625		1.7	160	267.8			
2490	36.4	0.1	0.9999	36.4	10	364.1	362.1	8.5	2.3
	18.1	0.05		18.1	20	362.5			
	8.7	0.025		8.7	40	349.6			
	4.5	0.0125		4.5	80	361.1			
	2.3	0.00625		2.3	160	373.4			
2491	17.1	0.1	0.9993	17.1	10	170.5	197.5	37.3	18.9
	9.0	0.05		9.0	20	179.5			
	4.4	0.025		4.4	40	175.5			
	2.5	0.0125		2.5	80	201.0			
	1.6	0.00625		1.6	160	261.0			

Three samples with higher titers were evaluated for linearity over the 1:20 – 1:320 dilution range and a standard curve ranging from 3.9 – 500 ng IgE/mL. The results are shown in Table 11.

Table 11: Linearity of Sample Dilution (b)

Sample	Mean IgE (ng/mL)	Dilution	R2 Value	Mean IgE (ng/mL)	Dilution factor	Adjusted IgE (ng/mL)	Mean	SD	CV%
2505	134	0.05	0.9989	134	20	2680	2443	153	6.2
	63	0.025		63	40	2509			
	29	0.0125		29	80	2330			
	14	0.00625		14	160	2320			
	7	0.003125		7	320	2375			
2508	177	0.05	0.9998	177	20	3542	3401	149	4.4
	89	0.025		89	40	3570			
	42	0.0125		42	80	3347			
	21	0.00625		21	160	3322			
	10	0.003125		10	320	3223			
2510	112	0.05	0.9992	112	20	2237	2275	80	3.5
	60	0.025		60	40	2417			
	28	0.0125		28	80	2258			
	14	0.00625		14	160	2240			
	7	0.003125		7	320	2224			

Inter-Assay Variability

To evaluate inter-assay variability, five (5) human serum samples were run in duplicate on four different days. The IgE assay was performed as previously described in the Methodology section

with a standard curve ranging from 3.9 – 500 ng/mL, samples tested at a 1:10 dilution in block buffer, and a conjugate dilution of 1:500. The IgE titers for these four assays are shown in Table 12 below. The assay exhibited good reproducibility over the four assays with CV% ranging from 6.0 – 15.4%.

Table 12: Total IgE – Inter-Assay Variability

	Run	Run	Run	Run	Mean		
Sample #	1	2	3	4	IgE (ng/mL)	SD	CV%
2498	112	84	97	109	100	13	12.8
2499	282	252	295	337	292	35	12.2
2500	95	67	75	85	80	12	15.4
2501	1533	1688	1726	1799	1686	112	6.7
2502	47	45	44	50	46	3	6.0

Intra-Assay Variability

To evaluate intra-assay variability, five (5) human serum samples were run in duplicate in sets of three (3) replicates. The IgE assay was performed as previously described in the Methodology section with a standard curve ranging from 3.9 – 500 ng/mL, samples tested at a 1:10 dilution in block buffer, and a conjugate dilution of 1:500. The IgE titers are shown in Table 13 below. The assay exhibited excellent intra-assay reproducibility with CV% < 5%.

Table 13: Total IgE - Intra-Assay Variability

Sample #	Individual IgE titers (ng/mL)	Mean IgE	%CV
2498	84 83 80	82	2
2499	252 252 258	254	1
2500	67 67 70	68	3
2501	1688 1623 1632	1648	2
2502	45 45 46	45	2

Initial screen of a panel of pre- and post-1998 human serum samples

An initial screen of pre-1998 and post-1998 human serum samples was performed. These samples were available in the laboratory and had been used for validation of other ELISA assays, or tested as clinical samples. All samples were diluted 1:10 (higher dilutions were used for samples with limited volumes) and run in duplicates. As can be seen in Table 14 and Figure 3, there was a range in total IgE levels in the various individuals from both groups. This is consistent with the published literature (N. Franklin Adkinson Jr.,"*Measurement of total serum immunoglobulin E and allergen-specific immunoglobulin E antibody*", In: Manual of Clinical Immunology, 1980, second edition, p704-807 , and Dennis R. Ownby, *"Clinical Significance of IgE*" , In: Allergy: Principles and Practice, 1993, fourth edition, p 1059-1076).

Table 14 : Total IgE in Pre-1998 and Post-1998 Human Serum Samples

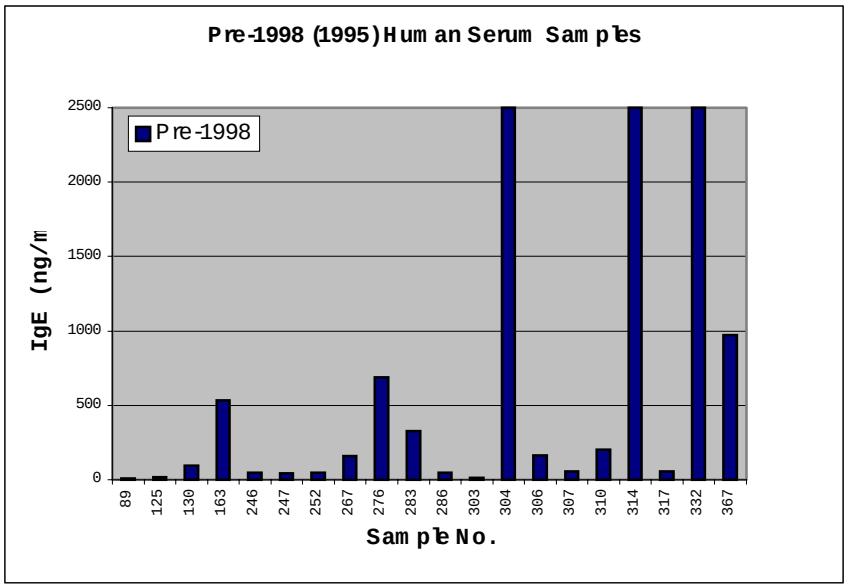
Pre-1998			**Post-1998**	
Sample	IgE (ng/mL)		Sample	IgE (ng/mL)
89	7*		2497	52
125	17*		2498	112
130	94		2499	282
163	533		2500	95
246	49		2501	1533
247	42		2502	47
252	48		2503	12*
267	161		2504	138
276	687		2505	2500**
283	325		2506	53
286	48		2507	1024
303	15*		2508	2500**
304	2500**		2509	148
306	165		2510	2500**
307	55		2511	31*
310	201		2512	58
314	2500**		2513	10*

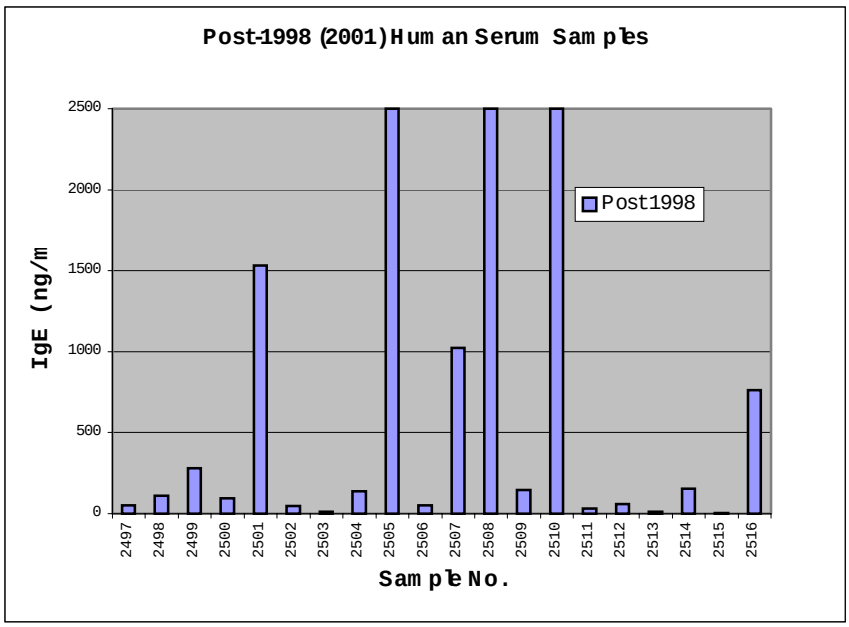
317	55		2514	154
332	2500**		2515	5*
367	971		2516	761
Mean:	245		Mean:	343
SD:	288		SD:	468
SE:	64		SE:	105

(*) < 39 ng/mL (Sample needs to be re-tested at a dilution less than 1:10)

(**) >2500 ng/mL (Sample needs to be further diluted and re-tested)

Figure 3: Total IgE in a Panel of Pre-1998 and Post-1998 Human Serum Samples





Evaluation of Peanut and Brazil Nut Allergic Individuals

Serum samples from five (5) peanut allergic, and one (1) brazil nut allergic individuals were provided by Dr. S. Hefle from the University of Nebraska. Each sample was diluted 1:10, 1:100, 1:1000, and 1:10,00 in block buffer and tested in duplicate. The assay and all data were performed as described in the Methodology section. The titers obtained (Table 15) ranged from 170 –9351 ng/mL total IgE.

Table 15: Total IgE in Individuals with Nut Allergies

Sample #	Dilution	IgE (ng/mL)	IgE (ng/mL)
1P	1:10 1:100 1:1000 1:10000	459 542 * *	501
2	1:10 1:100 1:1000 1:10000	237 * * *	237
3	1:10 1:100 1:1000 1:10000	* 9203 9499 *	9351
4	1:10 1:100 1:1000 1:10000	1371 1233 * *	1302
5	1:10 1:100 1:1000 1:10000	170 * * *	170
6	1:10 1:100 1:1000 1:10000	1297 1289 * *	1293

** - Sample out of Range*

4.5 Human anti-Cry9C-Reactive IgE

The anti-Cry9C-specific IgE ELISA was based on the previous work done with the goat, rabbit, and human Cry9C-specific IgG ELISA, using a coating concentration of Cry9C of 0.5 ug/well. The conjugated goat anti human IgE was used at a 1:500 dilution as in the total IgE ELISA. Samples were serially diluted 1:2 with starting dilutions of 1:2.5, and tested in duplicate. None of the human serum samples from the initial screen panel, nor from the nut-allergic individuals showed any reactivity compared to background. Thus, no anti-Cry9C-reactive IgE could be detected in any of the samples tested.

5.0 CONCLUSIONS

ELISA systems were developed to measure the levels of Cry9C-specific IgG antibody present in goat and rabbit polyclonal antibody preparations provided by Aventis CropScience. Background Cry9C reactivity was observed in both rabbit and goat normal sera obtained from commercial vendors. In both species, this reactivity was approximately 1000-fold less than that obtained from the animals immunized with Cry9C (polyclonal preparation). The human ELISA for Cry9C-reactive human IgG for both pre- and post-1998 sera also had background reactivity to Cry9C. The level of reactivity was variable and was comparable (means within 2 Standard Deviations). This low level of binding represents normal "background" since the StarLink™ corn containing the Cry9C protein had not been grown in the U.S. before 1998. Low levels of reactivity were also measured in nut-allergic sera.

The development of the Cry9C-specific IgE ELISA was preceded by the development of an ELISA for total serum IgE levels. The total IgE ELISA was used to demonstrate binding of the anti-human IgE conjugate to IgE in normal serum and assay sensitivity. The total IgE ELISA was a sensitive assay able to detect less than 1 ng/mL of human IgE with good reproducibility (both inter-assay, and intra-assay) and good linearity over at least a 10-fold dilution range. Total IgE titers were obtained for the panel of pre-1998 and post-1998 sera, and for the nut-allergic sera. Using the system optimized for the total IgE ELISA, and the Cry9C coating concentrations used to detect Cry9C-specific IgG, an initial panel of pre- and post-1998 samples was evaluated for both total IgE and Cry9C-specific IgE. Total IgE titers were detectable in all samples tested (in pre- and post-1998 sera, and nut-allergic sera). However there was no Cry9C-specific IGE reactivity in any of the samples tested.

The total IgE ELISA was developed using purified human IgE. This assay can detect concentrations of IgE less than 1 ng/mL. An IgE standard in International Units (IU) is being obtained to make these titers more comparable to those reported in the literature.

A panel of 100 (pre-1998) plasma samples has recently been obtained form the American Red Cross and will be tested in the three (3) human ELISA systems to determine baseline background values. Means and standard deviations will be obtained for (1) Cry9C-specific IgG, (2) Cry9C-specific IgE, and (3) total IgE. A positive for post-1998 sera could then be defined as a titer greater than 3 standard deviations from the100-sample group mean.